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Allogeneic Fibroblasts Used to Grow Cultured Epidermal Autografts Persist in Vivo and Sensitize the Graft Recipient for Accelerated Second-Set Rejection

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Introduction: **Cultured epidermal autografts (CEAs) have been used for wound coverage in patients with massive burns and other skin defects. However, CEAs often display late breakdown, which may be immunologically mediated and initiated by persistent foreign fibroblasts used as a feeder layer to optimize keratinocyte growth. This study investigates whether these fibroblasts, previously shown to persist in vitro, survive after grafting and induce host sensitization to alloantigen.**

Methods: **CEAs from CBA donors (H-2^k) were grown on allogeneic NIH 3T3 (H-2^q) or syngeneic LTK (H-2^k) fibroblasts, which were removed by trypsinization 7 days later. CBA mice (n = 85) were flank-grafted with NIH allografts (positive control), CEA/3T3s, CEA/LTKs, or CBA autografts (negative control). Hosts were challenged with second set NIH tail allografts 3 weeks later. Median graft survival was compared between groups by Wilcoxon rank and χ^2 analysis. Additional CBA mice (n = 15) received CEAs that were biopsied 0, 4, and 8 days after grafting. The presence of allogeneic fibroblasts was determined by Western immunoblotting, using KL295, a monoclonal antibody that recognizes H-2^q (but not H-2^k) class II histocompatibility antigens.**

Results: **Allogeneic fibroblasts persisted after grafting but decreased over time, as determined by alloantigen expression on Western immunoblots. Accelerated tail graft rejection occurred in hosts primed by NIH allografts (9 days, $p < 0.05$), as well as by CEAs grown with an allogeneic (10 days, $p < 0.05$) but not a syngeneic feeder layer (12 days, NS). Mice receiving flank autografts rejected second set tail allografts at 12 days.**

Conclusions: **Immunogenic fibroblasts used to grow CEAs survive in vivo and sensitize the graft recipient for accelerated second-set rejection. These persistent cells may initiate an inflammatory response that may result in late graft breakdown and limit the utility of CEAs grown with a foreign fibroblast feeder layer.**

Key Words: **Burn injury, Skin replacement, Cultured epidermal autograft.**

Cultured epidermal autografts (CEAs) have been proposed as a biologic, functional skin replacement in patients with massive thermal injury.[1–3] Wound coverage in these patients represents a significant technical problem, because of limitations in the quality and quantity of autogenous, partial-thickness skin that can be harvested. CEAs are particularly attractive because these grafts permit the 10,000-fold expansion of donor keratinocytes, but the 3-week cultivation period required for their growth, combined with their unpredictable take, their lack of a dermal component, and their excessive cost, have dampened enthusiasm for this biotechnology.[4,5] Furthermore, many investigators have described the phenomenon of late graft loss, which appears to be independent of mechanical and infectious causes and may have an immunologic component.[6,7] Clinically, previously healed wounds undergo blistering and ulceration, sometimes resulting in total autograft destruction.

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We have recently reported that growth-arrested, xenogeneic fibroblasts routinely used to accelerate keratinocyte growth in vitro survive after passage to secondary and tertiary cultures, as evidenced by flow cytometry and Western immunoblotting.[8] Such persistence occurs despite established techniques of feeder layer removal. The purpose of this study is twofold: (1) to determine if foreign fibroblasts used to cultivate CEAs persist in vivo after grafting, and (2) to determine if these fibroblasts sensitize the host to foreign histocompatibility antigens. This is clinically important because immunogenic fibroblasts could initiate an inflammatory response that induces local CEA destruction and, possibly, complete graft breakdown.

MATERIALS AND METHODS

Experimental Design

To determine whether fibroblasts used to cultivate CEAs persist in vivo after grafting, we first grew CBA keratinocyte sheets with either a syngeneic (LTK fibroblasts) or an allogeneic (3T3 fibroblasts) feeder layer. CEAs were then flank-grafted onto CBA hosts (n = 15) and biopsied 0, 4, and 8 days after application. Wound samples were assessed for the presence of fibroblast-specific class II histocompatibility antigens by Western immunoblotting. In the second series of experiments, we used a model of second-set rejection to determine whether or not allogeneic fibroblasts survive to prime CEA recipients for accelerated rejection of second-set

TABLE 1. Summary of keratinocyte and fibroblast haplotypes

	Murine Haplotype	
	H-2^k	H-2^q
Keratinocyte/graft donors	CBA	NIH Swiss
Fibroblast cell line	LTK	NIH 3T3

All graft recipients were CBA mice (H-2^k).

allografts. CBA hosts (n = 85) were randomized to receive NIH full-thickness (FT) allografts (positive control), CBA FT autografts (negative control), intraperitoneal (IP) 3T3 fibroblasts (putative positive control), or CBA CEAs grown on a syngeneic feeder layer (putative negative control) or an allogeneic feeder layer (experimental group). Three weeks later, mice were challenged with FT second-set tail allografts, which were observed for rejection. Our hypothesis was that animals exposed to FT allografts, allogeneic IP fibroblasts, and CEAs cultivated with an allogeneic feeder layer would mount a more vigorous rejection response to second-set allografts than groups not exposed to alloantigen initially.

Animal Protocols

Eight- to 10-week-old, 20 gram, female CBA mice (H-2^k haplotype) (Harlan, Inc., Indianapolis, Ind) were used as autograft donors and graft recipients. Age-, weight-, and gender-matched NIH Swiss mice (H-2^q haplotype) (Harlan) were used as allograft donors. Table 1 summarizes the haplotypes of the keratinocyte/graft donors and fibroblast cell lines used in these experiments. All animal protocols had previously been approved by the University of North Carolina Committee on Animal Research and conformed to experimentation standards established by the National Institute of Health.

Keratinocyte Cultures

CEAs were cultivated based on methods originally described by Rheinwald and Green.[9] Except where noted, all tissue culture media were obtained from the Lineberger Cancer Center (Chapel Hill, NC). Tail skin was harvested from CBA donors, cleaned with 70% ethanol, and stored overnight at 4°C in Dulbecco's modified Eagle media (DMEM) and 0.1% penicillin/streptomycin. After washing the tails in Hank's buffered saline solution (HBSS), the skin was incubated for 2 hours at 37°C in 0.25% trypsin (Sigma Chemical Co., St. Louis, Mo). The epidermis was peeled apart from the dermis and vortexed to generate a single-cell keratinocyte suspension. Syngeneic (mouse connective tissue L cells (LTKs) ATCC CCL 1.3, H-2^k haplotype) and allogeneic (NIH 3T3 Swiss albino embryo fibroblasts (3T3s) ATCC CRL 1658, H-2^q haplotype) fibroblasts were used as feeder layers and were growth-arrested by exposing these cells to 4 μg/mL mitomycin-C for 45 minutes at 37°C. Fibroblasts were washed three times with HBSS, liberated from tissue culture plates with 0.1% trypsin, and suspended in plating media consisting of DMEM and Ham's F-12, 5% fetal bovine serum (Hyclone Laboratories, Logan, Utah), insulin 5.0 μg/mL, transferrin 5.0 μg/mL, and hydrocortisone 0.4 μg/mL (all from Sigma), cholera enterotoxin 0.01 μg/mL (Schwartz Mann/ICN Biochemical, Costa Mesa, Calif), and amphotericin 5.0 μg/mL (E. R. Squibb and Sons, Princeton, NJ).

Keratinocytes (4×10^6 cells total) were co-cultured at a 2:1 ratio with fibroblasts (2×10^6 cells total) in 15 mL of plating media for 3 days. Epidermal growth factor 10.0 ng/mL (Collaborative Research, Bedford, Mass) was added to the plating media for subsequent media changes, which were performed every 2 to 3 days. One week after initial plating, LTK and 3T3 fibroblasts were removed by differential trypsinization (0.1% trypsin with 0.02% ethylenediaminetetraacetic acid (EDTA) exposure for less than 5 minutes, followed by gentle rinsing with HBSS). Cultured keratinocytes were then allowed to reach confluence, which typically took 2 weeks. Three groups of CEAs were created using the technique described above: CEA − 3T3 (the feeder layer was selectively trypsinized and visibly removed), CEA + 3T3 (the feeder layer was not trypsinized and therefore not removed), and CEA + LTK (the feeder layer was left intact). The appearance of the keratinocyte sheets was assessed by filtered light microscopy and documented photographically.

Grafting Procedure and Antigen Priming

After reaching confluence, CEAs were released from culture dishes with the enzyme dispase (Boehringer Mannheim, Germany), put on petroleum-lined gauze with the basal surface exposed, and placed on the left flank fascia of anesthetized CBA hosts. The surrounding, circumferential wound edge was undermined to accommodate the gauze-protected grafts, which were covered with a hydrophilic dressing (Vigilon, C. R. Bond, Berkeley Heights, NJ) for 1 week and protected with a stapled fabric bandage. Full-thickness allografts and autografts were harvested from NIH and CBA mice, respectively, washed in HBSS, debrided of adipose, placed directly onto the left flank fascia of CBA hosts, and secured by skin staples and a similarly applied bandage. A sixth group of CBA mice was exposed to alloantigen by priming hosts with an IP injection of viable, nongrowth-arrested 3T3 fibroblasts (5×10^5 cells in 0.5 mL of plating media).

Western Immunoblotting

The persistence of allogeneic fibroblasts in vivo was determined by assessing CEAs for the presence of alloantigen, via Western immunoblotting, using a monoclonal antibody specific for class II alloantigen.[10] Four days before grafting, CEAs were treated with recombinant murine interferon-γ 10,000 U/mL (Genentech, San Francisco, Calif) to enhance class II antigen expression. Grafted wounds were biopsied 0, 4, and 8 days after grafting with a 3-mm punch probe. Protein lysates were prepared by solubilizing biopsies in 200 μL of 0.01 M Tris-HCL, pH 7.4, 0.15 M NaCl, 0.5% NP-40, and 1 mM phenylmethyl sulfonyl fluoride (PMSF). Samples were briefly vortexed, ice incubated for 15 minutes, centrifuged for 15 minutes at 4°C, stored at −80°C, and standardized for protein concentration immediately before immunoblotting. Twelve percent sodium dodecyl sulfate (SDS) polyacrylamide gels were cast with a Bio-Rad Model 360 vertical cell and a Model 361 casting chamber. Molecular-weight markers and detergent-extracted specimens were separated on one-

dimensional gels and transferred to nitrocellulose, which was blocked with 1% bovine serum albumin in Tris-HCL (pH 8.0). Immunoblots were then incubated with KL295, a murine monoclonal antibody that recognizes and binds to the denatured 30-kd β-chain of H-2^q and H-2^b (but not H-2^k) class II histocompatibility antigens. After primary antibody incubation, immunoblots were exposed to goat, anti-mouse, alkaline phosphatase-labeled, secondary antibody and developed to reveal protein bands.

Video Densitometry

Expression of fibroblast-specific, H-2^q alloantigen was quantified with video densitometry. Using Macintosh video recording equipment (Apple Computer, Cupertino, Calif), Western immunoblots were recorded as video images in PowerPoint (Microsoft, Redmond, Wash) and analyzed with Gel Capture software (NIH, Bethesda, Md). Class II alloantigen expression, as detected by the H-2^q/H-2^b specific KL295 monoclonal antibody, was objectively assessed by determining the pixel density for each protein band at 30 kd.

Second-Set Rejection

To test the immunogenicity of a persistent allogeneic feeder layer, the mice were challenged with second-set tail allografts, via a method previously reported.[11] Three weeks after IP priming with 3T3s (n = 16) or flank grafting with NIH FT (n = 16), CBA FT (n = 15), CEA + 3T3 (n = 10), CEA − 3T3 (n = 15), or CEA + LTK (n = 13) grafts, anesthetized mice received a 10-mm FT skin allograft (NIH) placed on the dorsal tail surface. This allograft was compared to a distally placed 10-mm FT skin autograft (CBA), which served as an internal control. Tail grafts were protected from organic debris and mechanical disruption by specially manufactured cylindrical glass tubes, which were removed after 3 days. Tail graft viability was assessed daily by two independent observers. Criteria for graft rejection were based on graft color, hair orientation, and scale integrity.

Statistical Analysis

Median survival time (MST) of second-set tail allografts was determined for each of the six groups and compared between groups by Wilcoxon rank and χ^2 analysis. Bonferroni's procedure was used to correct for multiple comparisons between groups. Statistical significance was assumed for differences of $p < 0.05$.

RESULTS

Human CEA Assessment

Our laboratory became interested in the hypothesis that immunogenic fibroblasts contribute to late CEA "rejection" based on our clinical observation that many patients at the North Carolina Jaycee Burn Center experienced late CEA breakdown, apparently independent of mechanical disruption or graft infection. To support and accelerate keratinocyte growth, human CEAs had been prepared with a murine 3T3 feeder layer, subsequently removed via differential trypsinization, using methods nearly identical to those of Rheinwald and Green.[9] Several weeks after successful CEA application and wound healing, many patients developed an inflammatory response that resulted in focal areas of graft blistering and ulceration, and occasionally complete CEA loss (Fig. 1). This phenomenon, combined with the significant cost of this technology, as well as the unpredictable initial graft take, prompted our laboratory to suspend the clinical use of CEAs until further investigation had elucidated the mechanism for breakdown and developed improved techniques of CEA cultivation.

Light Microscopy

In this experiment, murine CEAs were grown with either a syngeneic (LTK) of allogeneic (3T3) feeder layer. Figure 2 represents the appearance of a CEA sheet 7 days after initial plating; the keratinocyte monolayer consists of flat, cobblestone-type cells, while persistent fibroblasts are identified as raised, spindled-shaped cells found clustered as islands throughout the monolayer. Three days after differential trypsinization, or 10 days after plating, nearly all of the feeder layer fibroblasts have been removed, and keratinocyte sheets have reached confluence (Fig. 3). However, careful inspection of these sheets by light microscopy reveals a second population of cells, which appear to represent persistent allogeneic fibroblasts that could be transferred to the CEA recipient (Fig. 4).

Western Immunoblotting

To determine if these feeder layer fibroblasts persisted in vivo after grafting, we first cultivated CEAs with a feeder layer that was allogeneic to both the keratinocyte donor, as well as graft recipient. Western immunoblotting was performed to detect the presence of alloantigen in wound biopsies 0, 4, and 8 days after grafting. Alloantigen from 3T3 fibroblasts was detected using KL295, a murine monoclonal antibody that is specific for murine H-2^q (but not H-2^k) class II histocompatibility antigens.[10] As expected, CEAs grown with a 3T3 feeder layer that was not removed (CEA + 3T3) express a 30-kd band (2367 pixel units) in vitro that represents class II antigen from allogeneic fibroblasts (Fig. 5). Slightly diminished alloantigen expression is observed in CEAs where the 3T3 feeder layer has been visibly removed via trypsinization (1902 pixel units). Class II alloantigen expression decreases in vivo after grafting (4 days, 820 pixel units; and 8 days, 699 pixel units), but remains substantially greater than background nonspecific binding to syngeneic antigen of CBA splenocytes (266 pixel units). This suggests that viable, allogeneic fibroblasts survive after grafting, decrease over time, and are potentially immunogenic to the host as they express class II histocompatibility antigens. Positive controls, representing known class II alloantigen expression, include NIH splenocytes (10390 pixel units) and 3T3 fibroblast sheets incubated with interferon-γ (9304 pixel units). Multiple immunoblots from separate graft recipients were created and revealed similar results.

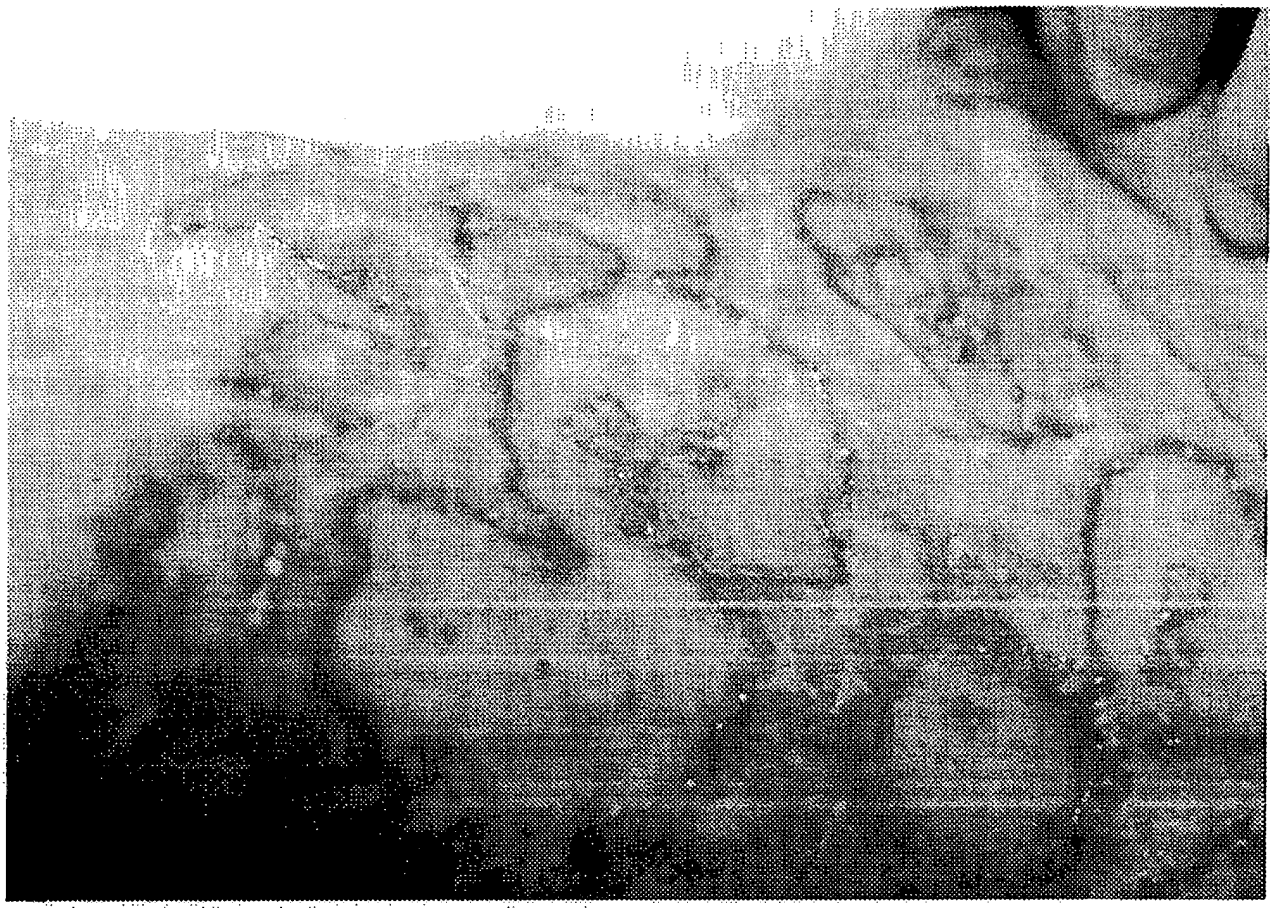

FIG 1. Appearance of late CEA breakdown, 20 days after grafting. The patient's left inguinal region is located in the upper right corner of the photograph. An intact portion of the CEA can be found along the patient's medial left thigh. Three weeks after successful engraftment, CEAs underwent spontaneous blistering and ulceration, with central areas of focal breakdown, as well as peripheral areas of advancing erythema. Within 48 hours, this portion of the CEA was completely destroyed, leaving only granulation tissue.

Second-Set Rejection

With evidence that feeder layer fibroblasts survive in vivo after grafting, we asked if such persistence resulted in host sensitization to alloantigen. To test the immunogenicity of CEAs grown with allogeneic fibroblasts, we used a model of second-set rejection, in which hosts previously exposed to alloantigen will generate a more vigorous rejection response when reexposed to that specific alloantigen.[12] In our particular experiment, we hypothesized that mice previously exposed to an allogeneic feeder layer would reject FT allografts

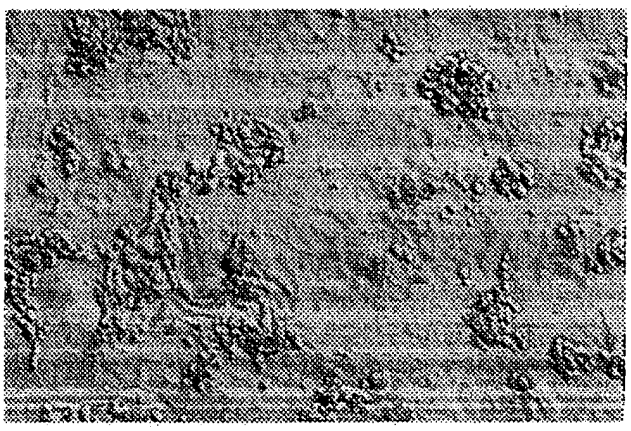

FIG 2. Photomicrograph of keratinocyte sheet, 7 days after plating. At least two populations of cells can be identified: the keratinocyte monolayer, which has a cobblestone-like, flat appearance, and residual feeder layer fibroblasts, which are spindle-like and raised relative to the keratinocytes. Magnification: 100×.

FIG 3. Photomicrograph of keratinocyte sheet, 10 days after plating and 3 days after differential trypsinization. The fibroblast feeder layer has been visibly removed, and the cultured keratinocytes have reached almost 100% confluence. Magnification: 100×.

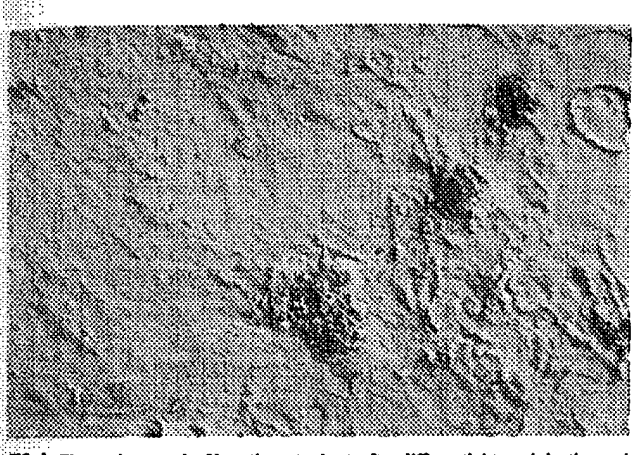

FIG 4. Photomicrograph of keratinocyte sheet, after differential trypsinization and before grafting. Although nearly all of the fibroblasts have been removed from this keratinocyte sheet, careful inspection by light microscopy reveals a second population of cells, which appear to be persistent fibroblasts surviving to engraftment. Magnification: 100×.

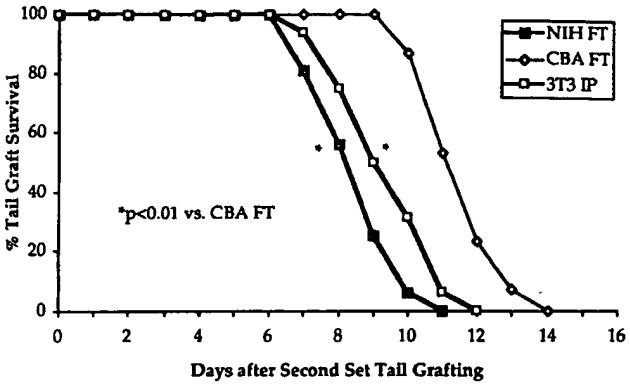

FIG 6. The effect of antigen priming on second-set rejection. H-2^k hosts primed with H-2^q alloantigen from both NIH full-thickness allografts and intraperitoneal 3T3 fibroblasts reject second-set tail allografts more vigorously than hosts grafted initially with syngeneic CBA skin ($p < 0.01$). Abbreviations: FT, full-thickness; IP, intraperitoneal.

more quickly than mice originally grafted with CEAs grown on syngeneic fibroblasts.

To investigate this query, we first had to characterize the effect of alloantigen priming, by observing the second-set rejection response of positive controls (NIH FT flank grafts), negative controls (CBA FT flank grafts), and putative positive controls (3T3 fibroblasts injected IP). Survival curves of second-set tail allograft survival are depicted in Figure 6. Mice primed with NIH FT flank grafts and IP 3T3 fibroblasts rejected tail allografts at 9 and 9.5 days, respectively, compared to mice flank grafted with autografts, who rejected second-set tail allografts after 12 days ($p < 0.01$). There was no statistical difference between the NIH FT and 3T3 FB groups. Median survival time (MST) of tail allografts, MST range, and sample size are listed in Table 2.

Three experimental CEA groups (CEA + 3T3, CEA − 3T3, and CEA + LTK) were studied to determine whether or not the persistent feeder layer sensitizes the host to alloantigen. Mice grafted with CEA + LTK rejected second-set grafts at 12 days, with a tail graft survival curve nearly identical to mice receiving the flank autografts (Fig. 7). However, mice grafted with CEA + 3T3 and CEA − 3T3 demonstrated accelerated tail graft survival (9 and 10 days, respectively), compared to the negative control of 12 days ($p < 0.01$). Although the second-set survival curves suggest that CEA + 3T3 grafts may be more immunogenic than CEA − 3T3 grafts, the difference between these groups, and compared to the positive controls, is not statistically signifi-

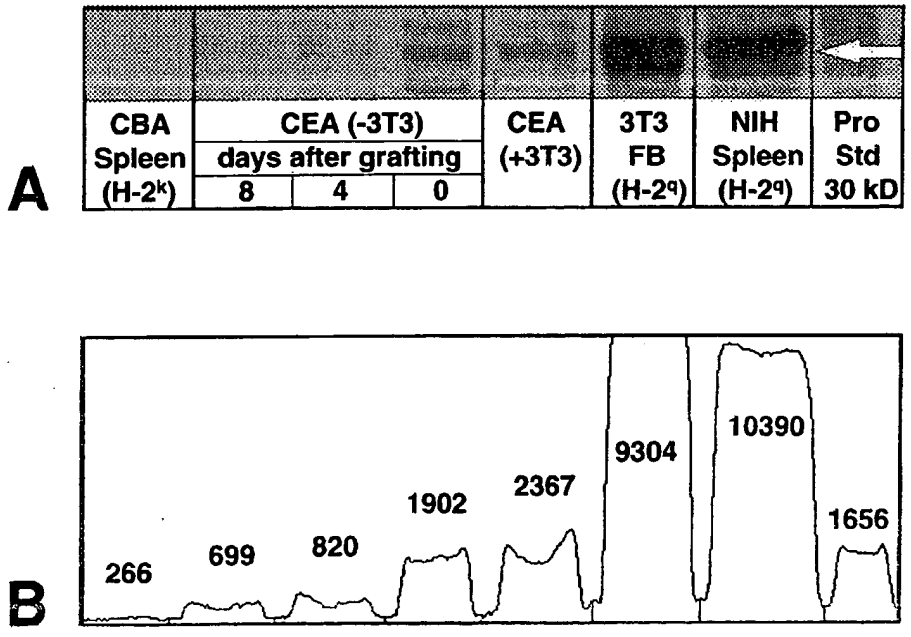

FIG 5. (*A*) Western immunoblot and (*B*) video densitometry depicting H-2^q class II antigen expression. Positive controls (NIH splenocytes and 3T3 fibroblasts, both H-2^q) express a 30-kd protein band (*arrow*) which represents the β-chain of H-2^q class II histocompatibility antigen. CEAs (H-2^k) co-cultured with 3T3s express significant H-2^q class II antigen both before and after differential trypsinization. H-2^q antigen, derived from 3T3s, persists in vivo after grafting but decreases after 8 days. Negative control CBA splenocytes (H-2^k) do not express any H-2^q class II antigen. Abbreviations: Pro Std, protein standard; FB, fibroblast; CEA, cultured epidermal autograft.

TABLE 2. Median survival times of second-set tail allografts

	Control Groups			Cultured Epidermal Autografts		
	NIH FT Allograft ($H\text{-}2^q$)	CBA FT Autograft ($H\text{-}2^k$)	3T3 IP ($H\text{-}2^q$)	CBA CEA +3T3 ($H\text{-}2^q$)	CBA CEA −3T3 ($H\text{-}2^q$)	CBA CEA +LTK ($H\text{-}2^k$)
MST, days	9*	12	9.5*	9*	10*	12
MST, range	7–11	10–14	7–12	7–11	7–12	10–14
N	16	15	16	10	15	13

MST, median survival time; N, number/group; FT, full-thickness; IP, intraperitoneal; CEA, cultured epidermal autograft.
* $p < 0.01$ vs. CBA FT, CEA + LTK groups.

cant. These data strongly imply that an allogeneic feeder layer, even when removed by conventional techniques, persists in vivo after CEA grafting, primes the host to alloantigen, and sensitizes for accelerated second-set rejection.

DISCUSSION

In this series of experiments, we provide evidence that allogeneic fibroblasts used to cultivate CEAs persist both in vitro and in vivo, despite initially inhibiting these fibroblasts with mitomycin-C and selectively removing them via differential trypsinization. Furthermore, we demonstrate that alloantigen from foreign fibroblasts can be detected in CEAs 8 days after application. Although foreign antigen from these fibroblasts apparently decreases over time, hosts are nonetheless sensitized to alloantigen and demonstrate accelerated rejection of second-set allografts.

Cultured keratinocyte grafts have been used for burn wound coverage for nearly 15 years, but no clinical trials have overwhelmingly proven the advantage of this biotechnology over more conventional methods of wound closure.[3–6,13] CEAs provide a potentially unlimited source of autologous epidermal cells and would appear to be an attractive solution to the problem of massive thermal injury and limited donor sites. However, graft preparation requires a cultivation period of 3 weeks,[14,15] delaying wound excision and coverage and potentially mitigating the beneficial effects on host immunocompetence and survival.[16–20]

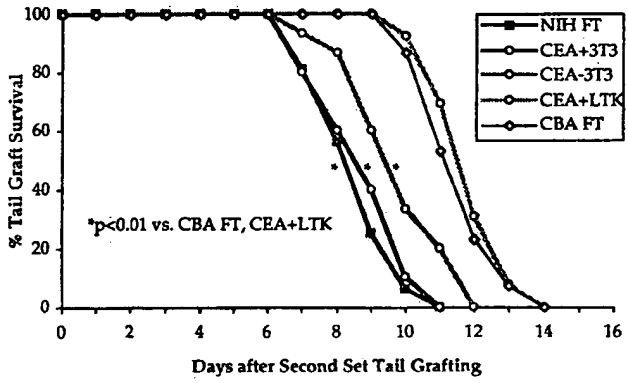

FIG 7. The effect of an allogeneic feeder layer of second-set rejection. CEAs cultivated with an allogeneic $H\text{-}2^q$ feeder layer (both removed and left intact) prime hosts for accelerated rejection of second-set tail allografts, compared to hosts receiving CEAs grown with a syngeneic $H\text{-}2^k$ LTK feeder layer ($p < 0.01$). Abbreviations: FT, full-thickness; CEA, cultured epidermal autograft.

Another significant limitation of CEAs is graft fragility, which is owing not only to the lack of a dermal component, but also abnormal anchoring fibrils located in the basement membrane. These disorganized attachments occur in older, mature grafts, despite the formation of a neodermis.[21] Such fragility interferes with patient rehabilitation by delaying the onset of physical therapy and the application of compression garments. Other investigators are discouraged by CEAs because of their unpredictable initial graft take, requiring multiple operative applications to complete wound closure.[4,6] The significant expense of these grafts, estimated to be $13,000 per % total body surface area covered, also presents a problem for cost-effective wound resurfacing.[6]

In addition to the concerns listed above, several authors have reported the phenomenon of late graft loss, which occurs independently of graft infection or mechanical factors.[6–8] Clinically, patients develop an inflammatory response that has been described as "rejection," in which graft blistering results in multifocal destruction, ulceration, and occasionally complete graft loss. Rue and Pruitt noted that six of ten patients with >70% total body surface area burn sustained significant, late CEA loss that appeared to be immunologically mediated. We were initially concerned that xenogeneic proteins used as media supplements in keratinocyte cultures (fetal bovine serum (FBS), bovine pituitary extract) might persist as antigens on autogenous keratinocytes, sensitize the graft recipient, and initiate autograft destruction.[22,23] While we discovered that patients grafted with CEAs generate antibodies to FBS, we did not examine the correlation between late CEA breakdown and anti-FBS antibody titers.

However, another source of immunogenic protein may be the foreign fibroblasts used to enhance keratinocyte growth in vitro. Several investigators have proposed that persistent fibroblasts may interfere with functional studies of epidermal grafts[24] and even transmit oncogenes to the graft recipient.[15] Certainly, there is reasonable concern that xenogeneic fibroblasts, if not completely removed from CEAs before grafting, might initiate an antibody- and/or cell-mediated immune response that could jeopardize the fate of the entire graft.[4,15,25] Using both flow cytometry and Western immunoblotting, we recently reported that murine fibroblasts persist in human keratinocyte cultures after several passages and express significant amounts of class II histocompatibility antigens.[8] Based on the methodology of Rheinwald and Green,[9] CEAs had been generated by plating keratinocytes with a growth-arrested feeder layer that was later removed via differential trypsinization. Commercially available CEAs differ only in that these grafts have xenogeneic fibroblasts added to secondary and tertiary cultures to improve keratinocyte growth, according to the patent application. Fibroblasts may not be required to grow keratinocytes in vitro, but adding fibroblasts to epidermal cells in culture clearly accelerates confluence[15] and remains the standard practice of private industry in the generation of CEAs.

The focus of this study was to determine if these foreign

fibroblasts survived after CEA application to sensitize the host immunologically. To test whether or not feeder layer fibroblasts persist in vivo, we chose to grow murine keratinocytes from genetically pure donors (CBA mice, H-2^k haplotype) with allogeneic murine fibroblasts (3T3, H-2^q haplotype). CEAs were then grafted onto CBA hosts after reaching confluence. Western immunoblotting revealed the presence of class II alloantigen up to 8 days after grafting, despite employing conventionally accepted techniques of feeder layer removal. These results are consistent with our previous finding that such fibroblasts persist in vitro after several passages.[8] Fibroblasts in these grafts remain viable, can express considerable class II histocompatibility antigen, and may account for 3% of the final cell population.[8]

Quantifying long-term CEA survival in a murine model is difficult, because of the rapid wound contraction that occurs in mice. Therefore, to assess the immunogenicity of persistent alloantigen, we utilized the concept of second-set rejection, as originally described by Medawar.[12] We found that mice initially primed with FT allografts, IP 3T3 fibroblasts, or CEAs grown with 3T3s (independent of their removal) sensitized the hosts to alloantigen and resulted in the accelerated rejection of second-set tail allografts (compared to mice grafted with FT autografts or CEAs grown with syngeneic LTK fibroblasts). Selective removal of the allogeneic feeder layer resulted in slightly prolonged second-set allograft survival compared to the positive control, but this difference was not statistically significant and still resulted in a more vigorous second-set rejection response, compared to the negative control.

How trypsinized CEAs sensitize the host remains speculative, but most likely involves either the persistence of nonvisible 3T3s in vivo or the persistence of soluble antigen in the graft matrix. Although this represents a minimal amount of foreign antigen, a critical amount must remain in vivo, to be detected by Western immunoblotting 8 days after grafting. One would suspect that if enough antigen can be detected by such methods, then this same amount of foreign protein could sensitize the host immunologically and prime for accelerated second-set rejection. Our particular model, which involves an allogeneic system, is undeniably different from the human situation, in which keratinocytes are cultured with xenogeneic fibroblasts. However, we are confident that the alloantigen priming demonstrated in these experiments can be extended to xenogeneic sensitization. The ability to demonstrate such priming between two similar murine haplotypes implies that the greater histocompatibility mismatch between the human host and mouse fibroblast will yield at least equivalent sensitization to foreign antigen.

The delayed destruction of CEAs observed in some graft recipients may occur only after restoration of host immunocompetence. We have previously demonstrated that burn injury impairs alloantigen processing as a function of burn size.[26] Sensitization to alloantigen and the effector mechanisms designed to eliminate foreign antigen are both impaired following thermal injury. Therefore, patients may only generate an immunologic response to foreign fibroblasts in CEAs after wound closure, improvement in nutritional parameters, and restoration of immunocompetence. However, once priming has occurred, focal destruction directed at xenoantigens could possibly induce a generalized inflammatory response that results in total graft breakdown. Why some patients do not display this phenomenon can be explained by two possibilities: (1) the successful removal of CEA fibroblasts before grafting, or (2) the development of chimeric tolerance during host immunosuppression. Clearly, additional clinical trials are necessary to determine whether graft recipients develop antibodies to xenoantigen and/or generate a cytotoxic T lymphocyte response.

We conclude that the persistence of foreign fibroblasts in keratinocyte cultures limits the clinical utility of CEAs when used for wound coverage. Patients who require multiple applications, such as those with massive burn injury, may be particularly susceptible to antigen sensitization and, therefore, late CEA breakdown. While keratinocytes can be cultivated without the use of a fibroblast feeder layer, the addition of such cells clearly accelerates culture time and improves culture quality, perhaps through the production of eicosanoids, extracellular matrix, growth factors, or stimulatory cytokines.[4,15,27,28]

We are presently investigating alternative models of keratinocyte cultivation, which include the addition of potentially mitogenic, synthetic antibiotic peptides to the culture media, as well as the use of a plastic monomer that allows for the transfer of subconfluent keratinocytes, as early as 4 to 7 days after initial plating, to freshly excised wounds. However, if the presence of a fibroblast feeder layer proves to be essential for optimal keratinocyte expansion, then perhaps the insertion of a "suicide gene," designed to induce fibroblast apoptosis, may prove to be an effective method of feeder layer removal. Undoubtedly, such genetic manipulation, even if successful in eliminating foreign antigens, would only add to the cost of an already expensive technology. Improving the initial graft take, however, would decrease the need for successive applications and may actually decrease the overall cost of wound coverage.

The addition of autogenous fibroblasts to epidermal cultures might accelerate keratinocyte growth and improve graft quality, but current cell culture technology does not easily permit the creation of an immediately available fibroblast cell line.[29] Nevertheless, the incorporation of a dermal component remains critical to the success of cultured grafts. Composite skin substitutes utilizing autologous keratinocytes and fibroblasts seeded onto collagen substrates have been successful in recreating both epidermal and dermal elements but have not yet been studied extensively in clinical trials.[30]

Despite the inconsistent success of CEAs, cultured keratinocyte sheets are probably effective as a biologic dressing, capable of secreting growth factors and providing residual adnexal elements, such as sweat glands and hair follicles, the opportunity to repopulate the wound surface.[4] However, until CEAs can be grown without an immunogenic feeder layer and demonstrate clinical usefulness, we remain cautious about the widespread application of CEAs for burn wound coverage.

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DISCUSSION

Dr. David G. Greenhalgh (Cincinnati, Ohio): Dr. Hultman has presented another fine work from Dr. Meyer's laboratory that examines the immunologic consequences of using cultured epidermal autografts. The well-designed study examines the causes of late loss of cultured epidermal autografts in burn patients. The hypothesis is that the feeder layers of fibroblasts that are cocultured with the cultured epidermal autografts persist despite attempts to separate the fibroblasts from the cultured epidermal cells. The persistent fibroblasts sensitize the host and ultimately lead to loss of the cultured epidermal autograft.

The authors present convincing data that support the hypothesis, but I do have the following questions. The Western blot is a very sensitive test, but is it overly sensitive? It would be interesting to examine the wound by immunohistochemistry to see if indeed there are intact fibroblasts persisting in the autografts.

In a similar line using histology, is there an inflammatory reaction showing evidence of rejection in the mice at the time of rejection of their autografts?

The authors have shown pictures of the patients losing their cultured epidermal autografts. Have there been biopsies performed looking at histology for evidence of rejection in these patients?

I agree that many makers of skin substitutes use feeder layers from an allogeneic source. Since it takes 3 weeks for the cultured epidermal autograft to be prepared, why not use the patient's own fibroblast as a feeder layer? Even more simply, why not culture the keratinocytes in a fibroblast free

media, such as has been used on a regular basis by Dr. Boyce and other investigators?

Dr. Charles Cuono and later many other investigators, including Dr. William Hickerson, have described the use of fresh allografts for a temporary coverage of the burn wound. After 3 weeks, when cultured keratinocytes are ready, the epidermal portion of the allograft is debrided, leaving an allograft dermis on the wound surface. These investigators have clearly shown that covering the dermal allograft with cultured epidermal autograft is well tolerated, and there does not appear to be a rejection problem in these patients. Can you explain why there is no rejection to the use of a dermal allograft when you show that a possible cause for late loss of cultured epidermal autografts may result from persistence of fibroblasts of the feeder layer. I am certain that there would be persistent allogeneic fibroblast in the allogeneic dermis used by these investigators.

I agree that cultured epidermal autografts alone have been quite ineffective. They are prone to persistent blistering problems and scarring problems. Our natural skin is composed of a dermis and an epidermis, and most burn surgeons now feel that both components need to be used for adequate wound coverage. Even the originators of the cultured epidermal autograft techniques now believe that a dermal component is important. Should our studies with the use of cultured keratinocytes alone be abandoned?

Finally, many investigators have shown that there are still persistent and significant problems with the use of skin substitutes, whether using epidermal or dermal components alone, or with a composite skin made up of both a dermis and an epidermis. While these problems persist, investigations such as the one presented here are extremely important, because I feel that, at one point, the use of skin substitutes will become the mainstay of the treatment of burn patients. Thank you.

Dr. John F. Hansbrough (San Diego, California): These studies were very interesting. We need to remember, though, that if you use 3T3 feeder cells, which we do not, they are murine cells, and therefore applying them to the human is a heterologous transplant, not allogeneic as in your studies, which is really perhaps entirely different in that these cells would probably be rejected by the host so quickly they may not have any effect on their response.

So again, the human situation would be a heterologous response, not allogeneic as in your studies.

Dr. Kevin T. Farrell (Allentown, Pennsylvania): There is a relatively small window, between 7 and 10 days, between when there is no loss and everything is rejected, and the question that I would have, we are putting a great deal of emphasis on the difference between 1 to 2 days rejection, are the criteria for rejection such that we do not have or observe a bias in commenting on this?

Dr. Basil A. Pruitt, Jr. (San Antonio, Texas): Is it possible to eliminate the allogeneic fibroblasts by exposure to a more concentrated solution of trypsin or exposure to the trypsin for a longer duration of time?

Secondly, in your clinical practice, have you noticed that with repeated applications of cultured cells there is greater and greater loss because of this phenomenon?

Thirdly, we know that with chimeric syngeneic, allogeneic constructs of cultured cells, there is retention and just gradual replacement of the allogeneic cells. How do you reconcile your findings with that phenomenon?

Dr. C. Sc tt Hultman (closing): Thank you very much for all of those comments. I will try to address each of the questions one by one.

First of all, Dr. Greenhalgh underscored the most important goal of our study, to create a permanent biologic skin replacement, which has properties that approach those of regular skin. Until we can perfect the dermal components of these cultured skin substitutes, we will be less than completely satisfied with these skin replacements.

Dr. Greenhalgh mentioned that the Western blot may not be the best assay to detect the presence of foreign protein, given its problems with specificity and sensitivity, but in our experiments, we found very little cross-reactivity with the $H\text{-}2^k$ antigen compared to the $H\text{-}2^q$ alloantigen. We are confident that the KL295 monoclonal antibody is not overly sensitive.

Secondly, Dr. Greenhalgh asked about immunohistochemistry, and we do not have any biopsy samples examining the persistence of these fibroblasts in vivo. But certainly that would be something we would want to pursue in future investigations.

Thirdly, Dr. Greenhalgh asked if we had obtained any biopsies of those patients who demonstrated autograft "rejection." The slide of the patient with autograft breakdown is actually 5 years old and does not have any accompanying histology. At that time, we suspected that this phenomenon was immunologically mediated, but we had no understanding of the mechanism. We abandoned the use of cultured epidermal autografts clinically and decided to focus our laboratory efforts on the basic science of trying to create a long-term skin replacement: accelerating keratinocyte growth in vitro, removing foreign antigens from the culture process, and studying the effect of burn immunosuppression on antigen processing.

Dr. Hansbrough expressed some concern that our model involved allogeneic fibroblasts, when in fact xenogeneic cells are used in commercially available skin grafts. In order to simplify our model, we chose to work with an entirely murine system. We felt that if we could show priming with allogeneic fibroblasts, then certainly the presence of xenogeneic fibroblasts would induce at least as strong a rejection response, if not greater, although we do not have the data to back that up.

Dr. Farrell asked about observer bias between the groups in terms of identifying second-set rejection of these tail grafts. We utilized a model that was reported several decades ago and represents a fairly standard approach to assess priming and sensitization, at least in the immunologic literature. Furthermore, we had two separate observers scoring the rejection response, and we have replicated our findings over the course

of several different studies. Our group is very confident that this particular model of second-set rejection is reliable and accurate.

And finally, to address Dr. Pruitt's questions, I think he raises an excellent, provocative concept of chimerism, which basically represents the incorporation of nonself into self through various immunologic mechanisms, either through tolerance or through host nonreactivity. Burn patients that are immunocompromised may have a higher propensity for chimerism than the immunocompetent host.

In this particular experiment, our hosts were immunocompetent, and were able to mount an immunologic response to these foreign fibroblasts. In burn patients, however, the prolonged survival of cultured epidermal allografts or autografts grown with foreign fibroblasts may be due to this chimerism that Dr. Pruitt has suggested. This might explain why many patients do very well with these cultured skin substitutes and do not have this rejection phenomenon that we have observed in a small subgroup of patients.

Finally, Dr. Pruitt asked whether or not prolonged trypsinization of cultured epidermal sheets might be more effective in removing the fibroblast feeder layer. In our experience, extended exposure to this enzyme damages the grafts and limits their effectiveness as a biologic skin replacement.

I think that wraps it up. Thank you very much.